

PCTWORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12Q 1/68, C07H 19/00, 21/00, 21/02, 21/04	AI	(11) International Publication Number: WO 97/43451 (43) International Publication Date: 20 November 1997 (20.11.97)
(21) International Application Number: PCT/US97/09094 (22) International Filing Date: 15 May 1997 (15.05.97) (30) Priority Data: 08/647,820 15 May 1996 (15.05.96) US (71) Applicant (for all designated States except US): BIOGENEX LABORATORIES [US/US]; Suite 200, 4600 Norris Canyon Road, San Ramon, CA 94583 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): SHENG-HUI SU, George [CN/US]; 9200 Alcosta Boulevard B-3, San Ramon, CA 94583 (US). IYER, Rajkumar, Siva [IN/US]; 7100 San Ramon Boulevard #11, Dublin, CA 94568 (US). AGGARWAL, Sunil, K. [IN/US]; 7100 San Ramon Road #8, Dublin, CA 94568 (US). KALRA, Krishan, L. [US/US]; 60 Eagle Ridge Place, Danville, CA 94506 (US). (74) Agent: WESEMAN, James, C.; The Law Offices of James C. Weseman, Suite 1600, 401 West A Street, San Diego, CA 92101 (US).	(81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i>	

(54) Title: NON-NUCLEOTIDE LINKING REAGENTS**(57) Abstract**

Non-nucleotide reagents capable of forming oligomers with nucleotide units are disclosed, together with intermediates for synthesizing such non-nucleotide reagents, oligomers incorporating such reagents, kits containing such reagents and methods for their use in forming oligomers with nucleotide units.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon		Republic of Korea	PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakhstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

-1-

DescriptionNon-Nucleotide Linking Reagents

5

Technical Field

The present invention relates generally to the use of non-nucleotide reagents as monomeric units in oligonucleotides.

10

Background of the Invention

In both research applications and clinical diagnosis, a known technique for determining the presence of a particular nucleotide sequence (the "target nucleotide sequence") in either RNA or DNA is to perform a nucleic acid hybridization assay. In such an assay, a nucleotide probe, typically an oligonucleotide, is selected having a nucleotide sequence complementary to at least a portion of the target nucleotide sequence. Typically, the probe is labeled to provide a means whereby the presence of the probe can be readily detected.

20

When the labeled probe is exposed to a sample suspected of containing the target nucleotide sequence, under hybridizing conditions, the target sequence will hybridize with such a labeled probe. The presence of the target sequence in the sample can then be determined qualitatively or quantitatively, usually after separating hybridized and non-hybridized probes and determining the presence or amount of the labeled probe which hybridized to the test sample.

25

Prior methods for linking a label to a nucleotide probe have generally utilized a single label attached to a nucleotide monomeric unit, and then incorporating one or more of the nucleotide monomeric units into the probe. For example, analogs of dUTP and UTP containing a biotin moiety have been chemically synthesized and incorporated into polynucleotides (P.R. Langer *et al.*, *Proc. Nat. Acad. Sci. USA* 78:6633 (1981)). Such biotin-labeled nucleotides may then be incorporated into nucleic acid probes of biological or synthetic origin.

30

Other methods for labeling nucleotide probes have been proposed which allow labels to be randomly linked to nucleotides in a nucleotide multimer. Numerous proposals have been made for incorporating multiple modified

-2-

nucleotides or non-nucleotide monomeric units into oligonucleotides with a view towards enhancing the detectability of the labeled probe and the target nucleotide sequence.

5 However, it has been demonstrated that use of such labeled nucleotides in a probe can reduce the stability of the hybrid formed with a target nucleotide sequence, particularly when multiple labels are present. Such reduced hybrid stability has been demonstrated for nucleic acid probes of biological origin possessing multiple biotin moieties, for synthetic oligonucleotides possessing multiple fluorescein labels, as well as for synthetic oligonucleotides possessing
10 biotin and fluorescein labels.

 In addition, derivatives of nucleotide linking phosphate groups have been disclosed, the nucleophilic moiety of which can be labeled following their incorporation into an oligonucleotide. However, such compounds, being based on nucleotide derivatives, would be expected to exhibit some of the disadvantages
15 discussed above for nucleotide based derivatives.

 More recently, 2-amino-1,3-propanediol structures have been used to label oligonucleotides with reporter groups (Nelsen, P.S. *et al.*, *Nuc. Acids Res.* **20**:6253 (1992)). However, these structures appear to demonstrate low coupling efficiency, and thus low yield of labeled oligonucleotides which furthermore must be carefully
20 purified before they can find use as probes for target sequences.

 Thus it is considered desirable to provide a non-nucleotide reagent which demonstrates high coupling efficiency and thus provides higher yield of labeled oligomer.

 Furthermore, it is also considered desirable to provide such a reagent which
25 will allow the resultant oligomers to anneal and hybridize with efficiencies approaching those of oligomers which contain only native nucleotide monomeric units.

-3-

Disclosure of the Invention

The present invention provides non-nucleotide reagents capable of forming an oligomer with nucleotide units, said reagents comprising compounds of the formula:

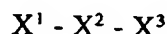


wherein

R^1 is selected from the group consisting of hydrogen, acid-sensitive, base-stable blocking groups and acyl capping groups;

X is selected from the group consisting of O, S, NH and N=N;

10 R^2 is a substituent group of the formula



wherein

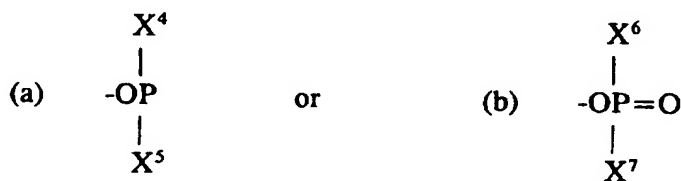
X^1 is a substituted or unsubstituted C_5 to C_7 cyclic moiety incorporating the carbon atom of the formula;

15 X^2 is selected from the group consisting of O, S, CH_2 , NH and N=N; and

X^3 is hydrogen or a linking functional group which is capable of linking with a functional moiety; and

R^3 is a linking group of the formula

20



25

wherein

X^4 is halogen or substituted amino,

X^5 is alkyl, alkoxy or phenoxy, or a cyano derivative thereof,

30 X^6 is halogen, amino or O, and

X^7 is alkyl, alkoxy or aryloxy, or may be H only if X^6 is O, or

R^3 is a bond, either directly or through an intermediate group, to a solid support.

Such reagents can be used to label or otherwise incorporate desirable functionalities into oligomers, utilizing conventional automated nucleotide synthetic protocols. The present reagents preserve the natural three carbon internucleotide phosphate distance, so as to preserve the hybridization and annealing properties of the nucleotide duplex.

Also provided in the present invention are intermediates useful for producing such non-nucleotide reagents, oligomers incorporating such reagents, kits containing such reagents and methods for use of the reagents in forming oligomers with nucleotide units.

Brief Description of the Drawings

Figure 1 is a schematic depiction of the synthetic protocol of Example 1(a), steps I and II;

Figure 2 is a schematic depiction of the synthetic protocol of Example 1(a), steps III, IV and V;

Figure 3 is a schematic depiction of the synthetic protocol of Example 1(b);

Figure 4 is a schematic depiction of the synthetic protocol of Example 1(c);
and

Figure 5 is a schematic depiction of the synthetic protocol of Example 1(f).

-5-

Detailed Description of the Invention

The present invention provides non-nucleotide reagents capable of forming an oligomer with nucleotide units, said reagents comprising compounds of the formula:

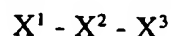


wherein

R^1 is selected from the group consisting of hydrogen, acid-sensitive, base-stable blocking groups and acyl capping groups;

X is selected from the group consisting of O, S, NH and N=N;

10 R^2 is a substituent group of the formula



wherein

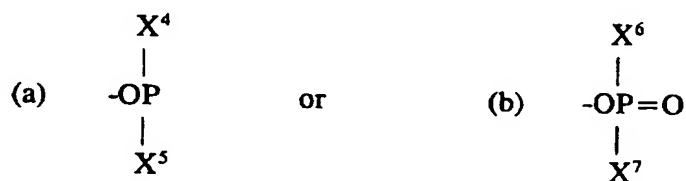
X^1 is a substituted or unsubstituted C_5 to C_7 cyclic moiety incorporating the carbon atom of the formula;

15 X^2 is selected from the group consisting of O, S, CH_2 , NH and N=N; and

X^3 is hydrogen, or a linking functional group which is capable of linking with a functional moiety; and

R^3 is a linking group of the formula

20



25

wherein

X^4 is halogen or substituted amino,

X^5 is alkyl, alkoxy or phenoxy, or a cyano derivative thereof,

30 X^6 is halogen, amino or O, and

X^7 is alkyl, alkoxy or aryloxy, or may be H only if X^6 is O, or

R^3 is a bond, either directly or through an intermediate group, to a solid support.

-6-

In the disclosure which follows, the following terms will have the indicated meanings unless a contrary meaning is otherwise apparent from the context in which the term is used.

5 As used herein, the term "nucleotide" is taken to mean a subunit of a nucleic acid consisting of a phosphate group, a five carbon sugar and a nitrogen-containing base. The term is also taken to include analogs of such subunits.

As used herein, the term "nucleotide oligomer" or "oligomer" is taken to mean a chain of nucleotides linked by phosphodiester bonds or analogs thereof.

10 As used herein, the term "nucleotide oligomer containing non-nucleotide monomers" is taken to mean an oligomer comprised of nucleotide units together with non-nucleotide monomeric units linked by phosphodiester bonds or analogs thereof.

15 The present invention provides a non-nucleotide reagent which can be coupled synthetically with nucleotide monomeric units to produce a defined sequence oligomer with a backbone comprised of nucleotide and non-nucleotide monomeric units.

In the formula first provided above,



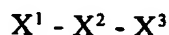
20 R^1 is a substituent group which is intended to be removed to facilitate linkage with other units in the backbone structure of a nucleotide oligomer containing non-nucleotide monomers. As such, R^1 is generally selected from the group consisting of hydrogen, acid-sensitive, base-stable blocking groups and acyl capping groups. Such groups are well known in the art, and include, for example, triphenylmethyl compounds, and alkoxy derivatives thereof, such as
25 dimethoxytriphenyl (DMT) groups.

The group identified as X functions in part to maintain proper intramolecular distance in the non-nucleotide reagent when functioning as a monomeric unit. Typically, X is selected from the group consisting of O, S, NH and N=N, although other atoms, or groups of atoms, could also serve in this capacity. Most
30 commonly, X will be O.

The group identified as R² is a substituent group which is intended to facilitate linkage with other functional moieties, and other functional groups, which may be desired to be included in a nucleotide oligomer containing non-nucleotide monomers.

5 Due to the chemical nature of the present non-nucleotide reagent, it may be positioned at any desired point within the nucleotide oligomer sequence. Thus it is possible to design a wide variety of properties into oligomers which contain both nucleotide and non-nucleotide monomeric units. Such properties include the attachment of specific moieties herein termed "functional moieties" at any desired location within the oligomer. Such moieties can include (but are not limited to) detectable labels (including enzymatic, fluorogenic, radioactive, chemiluminescent, and the like), intercalating agents, metal chelators, drugs, hormones, proteins, peptides, radical generators, nucleolytic agents, proteolytic agents, catalysts, specific binding agents (including biotin, antigens, haptens, antibodies, receptors, and the like), and other substances of biological interests, together with agents which modify DNA transport across a biological barrier, (such as a membrane), and substances which alter the solubility of a nucleotide multimer. Thus it is possible to position such labels and agents adjacent to any desired nucleotide.

The R^2 group is typically of the formula



wherein

X¹ is a substituted or unsubstituted C₅ to C₇ cyclic moiety incorporating the carbon atom of the formula;

25 X² is selected from the group consisting of O, S, CH₂, NH and N=N; and

X³ is a linking functional group which is capable of linking with a functional moiety.

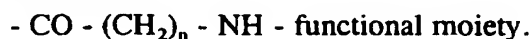
In the present reagent, the rigidity of the chemical structure of X¹ provides that
desirable feature of extending the linkage group and functional moiety away from
the oligomeric backbone structure, thereby substantially enhancing the coupling

-8-

efficiency of the reagents of the present invention. Commonly, X^1 will be substituted or unsubstituted cyclohexane.

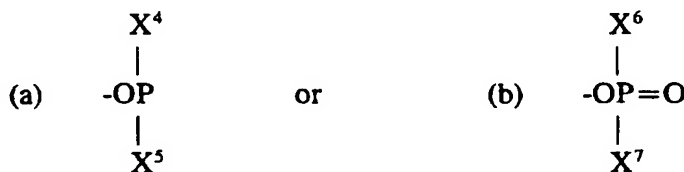
The group identified as X^2 functions as a linking and modifiable reactive group. Typically, X^2 is selected from the group consisting of O, S, NH, CH_2 , and N=N, although other atoms, or groups of atoms, could also serve in this capacity. Most commonly, X^2 will be NH.

In the formula, X^3 is hydrogen, or a linking functional group which can be of any length appropriate to the particular functional moiety selected. Typically, X^3 is a group of the formula



wherein n is an integer from 0 to 20. It is of course within the invention to add the functional moiety to the reagent prior to, or after, the inclusion of the reagent as a monomeric unit in an oligomer. In addition, the functional moiety can also serve as a bond to a solid support.

In the formula, R^3 is a substituent group which is intended to facilitate linkage with other units in the backbone structure of a nucleotide oligomer containing non-nucleotide monomers or to solid supports and the like. Typically, such linkage will be accomplished by automated methodologies, such as automated DNA/RNA synthetic protocols. As such, R^3 is generally selected from the group consisting of phosphodiesters, phosphotriesters, phosphites, phosphoramidites, H-phosphonates, alkyl-phosphonates, and phosphorothioates. Such groups are well known in the art, and include, for example, phosphorus linking group of the formula



wherein

X^4 is halogen or substituted amino,

X^5 is alkyl, alkoxy or phenoxy, or a cyano derivative thereof,

-9-

X⁶ is halogen, amino or O, and

X⁷ is alkyl, alkoxy or aryloxy, or may be H only if X⁶ is O, or
R³ is a bond, either directly or through an intermediate group, to a solid support.

5 As discussed above, the present non-nucleotide reagents will possess a linker
functionality to which desired chemical moieties have been or can be attached,
either prior to or after initiating the synthesis of the nucleotide oligomer.

10 In general, the techniques for linking moieties to the linker arm will be
similar to the techniques known for linking labels to groups on proteins. Examples
of useful chemistries include a reaction of alkyl amines with active esters, active
imines, aryl fluorides or isothiocyanates, and the reaction of thiols with maleimides,
haloacetyls, etc. (see generally Means, G.M. and R.E. Feeney, "Chemical
Modification of Proteins" Holden-Day Inc. (1971); R.E. Feeney, *Int. J. Peptide
Protein Res.* 29:145-161 (1987)).

15 As discussed above, due to the chemical nature of the present non-nucleotide
reagent, it may be positioned at any desired point within the nucleotide oligomer
sequence. Thus it is possible to design a wide variety of properties into oligomers
which contain both nucleotide and non-nucleotide monomeric units. Such
properties include the attachment of specific functional moieties at any desired
location within the oligomer.

20 Other benefits provided by the practice of the present invention include the
ability to immobilize the defined sequence to a solid support by employing the
linker arm functionality conjoined to a chemical moiety of the support in order to
construct, for example, nucleotide affinity supports. Multiple chemical moieties
can also be incorporated into the oligomer through multiple non-nucleotide
25 monomeric units in a particular nucleotide oligomeric sequence.

30 One can also provide oligomers which differ from naturally occurring
polynucleotides in that they include altered activities by utilizing proteins and
enzymes which act on polynucleotides. For example, the placement of the non-
nucleotide monomeric unit on the 3' terminus of an otherwise pure polynucleotide
will impart resistance to degradation by snake venom phosphodiesterases, or
providing specific cleavage sites for selected nucleases.

-10-

Hybridization probes may also be constructed by interspersing hybridizable nucleotide monomeric units and non-nucleotide monomeric units. For example, a mixed synthesis of nucleotide and non-nucleotide monomers can be performed whereby a defined sequence of nucleotide monomers are synthesized followed by a sequence of one or more non-nucleotide monomeric units, optionally followed by a second block of a defined sequence of nucleotide monomers.

The present invention also provides the ability to construct synthetic probes which simultaneously detect nucleotide multimers which differ by one or more base pairs. This can be accomplished by using the non-nucleotide reagents described herein to replace the nucleotides in a probe with non-nucleotide monomeric units at selected sites where differences occur in the nucleotide sequence of the various target nucleotide sequences.

In selected embodiments of the invention, labeled hybridization probes are constructed as oligomers with a defined sequence comprised of nucleotide and non-nucleotide monomers. Such non-nucleotide monomeric units can be grouped in a selected region or interspersed throughout the sequence of the nucleotide oligomer. The non-nucleotide monomeric units can be chemically labeled for use in hybridization reactions.

In the present invention, the non-nucleotide reagent is provided in a manner which permits it to be added in a stepwise fashion to produce a mixed nucleotide, non-nucleotide oligomer employing current DNA/RNA synthesis methods. Such reagents would normally be added in a stepwise manner to attach the corresponding monomeric unit to an increasing oligonucleotide chain which is covalently immobilized to a solid support. Typically, the first nucleotide is attached to the support through a cleavable ester link prior to the initiation of synthesis. In the present invention, the non-nucleotide reagent can be provided conveniently linked to such solid supports, for example, to controlled pore glass (CPG), to resins, polymers such as polystyrene, and the like. Stepwise extension of the oligonucleotide chain is normally carried out in the 3' to 5' direction. Such nucleic acid synthesis methods are provided, for example, in S.A. Narang, "Synthesis and

-11-

Applications of DNA and RNA," Academic Press (1987) and in M.J. Gait
"Oligonucleotide Synthesis," IRL Press, Washington, D.C. (1984).

When synthesis is complete, the oligomer is cleaved from the support by
hydrolyzing the ester linkage and the nucleotide originally attached to the support
becomes the 3' terminus of the resulting oligomer. Accordingly, the present
invention provides both a reagent for preparing oligomers which contain a mixture
of nucleotide and non-nucleotide monomeric units, together with methods for
utilizing such reagents in the construction of such oligomers.

Typically, the present reagents will possess two coupling groups so as to
permit the stepwise inclusion into a oligomer of nucleotide and non-nucleotide
monomeric units. The first of said coupling groups will have the property that it
can couple efficiently to the terminus of a growing chain of monomeric units. The
second of said coupling groups is capable of further extending, in a stepwise
fashion, the growing chain of mixed nucleotide and non-nucleotide monomers.
This typically requires that the second coupling group be inactivated while the first
coupling group is coupled, so as not to substantially couple at that time, the second
coupling group can thereafter be activated so as to then couple the non-nucleotide
monomeric unit. The inactivation is preferably accomplished with a protecting
group on the second coupling group, which can then be removed to activate the
second coupling group. It is also considered to be within the scope of the invention
that such "inactivation" and "activation" might be accomplished simply by changing
reaction conditions (e.g. pH, temperature, concentration of reagents, and the like)
with second coupling groups of suitable chemical structure which also lend
themselves to inactivation and activation by such techniques. Such coupling groups
permit the adjacent attachment of either nucleotide or non-nucleotide monomeric
units. It is considered desirable that such coupling groups operate through coupling
and deprotection steps which are compatible with standard automated DNA
synthesis methods.

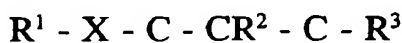
Such methods typically require that synthesis occur unidirectionally and that
all coupling cleavage and deprotection steps occur under "nonadverse conditions"

-12-

that is they do not substantially adversely effect the oligomer backbone and its various components.

Thus, the present invention provides oligomers containing the present non-nucleotide reagents, as well as methods for using such reagents in the synthesis of oligomers containing both nucleotide and non-nucleotide units.

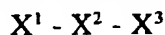
The invention further provides intermediates which are useful to synthesize the present non-nucleotide reagents. One embodiment of such an intermediate is provided by the formula:



wherein

R^1 is hydrogen; X is oxygen;

R^2 is a substituent group of the formula

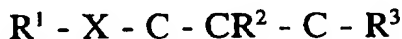


wherein X^1 taken together with the carbon atom of the formula is cyclohexane, X^2 is NH, and X^3 is H; and

R^3 is OH.

In this embodiment, the intermediate is of a structure similar to that of the present reagents, without having the functional groups included at R^1 , X^3 and R^3 .

In order to facilitate the use of the present reagents, kits for use in constructing oligomer can be provided to simplify practice of the method described above. The kit will typically contain a receptacle adapted to hold one or more individual reagent containers and at least a first container containing (1) a reagent in accordance with the formula



wherein

R^1 , X, R^2 , and R^3 are as previously defined. The reagent can be provided as a solution comprising a solvent and the reagent or (2) the reagent in an amount appropriate to make up the desired concentration when solvent from another container is used to fill the reagent container to a predetermined level.

-13-

In many cases, the kit will also contain at least a second container containing (1) a reagent used in the synthesis of oligomers, or (2) a reagent used in the detection of the functional moiety included in the subject reagent, or containers with both such materials. Such reagents are well known in the art and require no further description here. Specific examples are given in the general examples of the invention set out below. Appropriate instructions for carrying out the method of the invention will also be included in the kit.

The following examples serve to illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

Experimental

In the experimental disclosure which follows, all weights are given in grams (g), milligrams (mg), micrograms (μg), nanograms (ng), or picograms (pg), all amounts are given in moles (mol), millimoles (mmol), micromoles (μmol), nanomoles (nmol), picomoles (pmol), or femtomoles (fmol), all concentrations are given as percent by volume (%), proportion by volume (v:v), molar (M), millimolar (mM), micromolar (μM), nanomolar (nM), picomolar (pM), or normal (N), all volumes are given in liters (L), milliliters (mL), or microliters (μL), and linear measurements are given in millimeters (mm), or nanometers (nm) unless otherwise indicated.

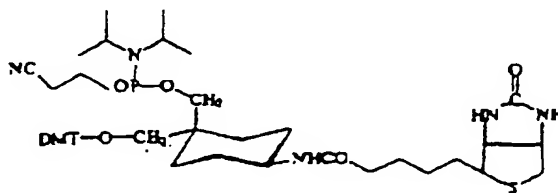
The following examples serve to demonstrate the synthesis of reagents of the present invention, as well as their use in forming oligomers with nucleotide units in accordance with the invention.

In the examples, the following abbreviations are used: "CX" is intended to refer to cyclohexane, "Bz" is intended to refer to benzoyl, "CED" is intended to refer to cyanoethyl N,N-diisopropyl phosphoramidite and "LC" is intended to refer to long chain.

Example 1

Reagents in accordance with the present invention can be synthesized in accordance with chemical synthetic techniques well known in the art. The following synthetic protocols demonstrate the synthesis of selected compound within the scope of the present invention.

- (a) Synthesis of Reagent compound 1 wherein R¹ is dimethoxytriphenyl (DMT), X is O, R² is cyclohexane - NH - CO - biotin, and R³ is phosphoramidite.

**[COMPOUND 1]**

The synthetic protocol for Compound 1 is outlined below and depicted in Figures 1 and 2:

Step I: Synthesis of BzO-CX

To an ice-cold solution of 500g 4,4-bis(hydroxymethyl)-1-cyclohexene in 3.0L of pyridine, was added dropwise 1.03L of benzoyl chloride. The reaction mixture was stirred at room temperature overnight, when TLC analysis (ethyl acetate/hexane 1:4 v/v) indicated the reaction to be complete. The reaction was quenched by the addition of 100mL water, followed by stirring at room temperature for 1 hour.

The reaction mixture was evaporated *in vacuo* to afford a syrupy residue. This was dissolved in methylene chloride and washed with 5% aqueous NaHCO₃.

-15-

solution. The organic solution was dried over anhydrous sodium sulfate, filtered, and concentrated *in vacuo* to afford 1,500g of crude product.

This product was purified by column chromatography over silica gel, using ethyl acetate/hexane (1:4, v/v) to elute the product (yield 1,050g). The desired product was dried under high vacuum for 2 days.

Step II: Synthesis of $\text{NH}_2\text{-CX}$

To a stirred solution of 120g of BzO-CX in 300mL diglyme, under argon, was added dropwise a solution of 5.5g NaBH_4 in 150mL of diglyme. The reaction mixture was slowly heated to 70°C . A solution of 23.4mL of $\text{BF}_3\text{-Et}_2\text{O}$ in 30mL diglyme was then slowly added to the reaction mixture and the resulting mixture stirred at 70°C for 1 hour. The reaction was quenched by the addition of 2.5mL water. This was followed by the addition of 50g of hydroxylamine-O-sulfonic acid and the reaction mixture was heated at 100°C for 3 hours. The reaction mixture was cooled to room temperature and then extracted into 1.2L of methylene chloride. The organic extract was washed with 500mL water, followed by 5% NaHCO_3 solution (2 X 300mL). The organic layer was dried over anhydrous sodium sulfate, filtered, and solvents removed by rotary evaporation to give 270g of crude product.

Purification of the product by silica gel column chromatography, using a solvent system comprising of 2.5-8.0% methanol in methylene chloride to elute the product, afforded 50g of pure 4-amino isomer of $\text{NH}_2\text{-CX}$. Small quantities of the undesired 3-amino isomer of $\text{NH}_2\text{-CX}$ were also formed.

Step III: Synthesis of BzO-CX-Biotin

To 13.5g of the amino compound $\text{NH}_2\text{-CX}$ obtained above, was added 200mL of methylene chloride. To the resulting solution was added 18.8g of Biotinyl N-hydroxysuccinimide ester (Biotin-NHSu) dissolved in 200mL of DMF. The reaction mixture was stirred for 15 min at room temperature, followed by the addition of 10.3mL triethylamine. The reaction was allowed to proceed for 1.5 hours, when TLC analysis revealed the reaction to be complete.

Methylene chloride was removed by rotary evaporation, and the resulting residue treated with 30mL methanol and with 25mL of 10% aqueous sodium carbonate solution. The solution was stirred at room temperature for 1 hour and

-16-

then extracted with 1.2L of ethyl acetate. The organic layer was washed with brine (2 X 400mL), dried over anhydrous sodium sulfate, filtered, and solvents removed *in vacuo* to finally give 22g of crude product.

5 This product was purified by column chromatography over silica gel, using gradient elution with 2.5-8.0% methanol in methylene chloride to yield 15g.

Step IV: Synthesis of DMT-CX-Biotin

10 To a stirred solution of 14.3g of BzO-CX-Biotin in 200mL DMF, was added 20mL of 25% sodium methoxide in methanol, and the resulting mixture stirred at 0-5°C for 1 hour. The pH of the solution was then adjusted to 7.0 by the addition of 60g Dowex 50X8-100 resin to the reaction mixture followed by stirring for 15 min. The resin was filtered off and the filtrate evaporated to remove DMF. The resulting residue was dissolved in 10mL methylene chloride and the product precipitated by the addition of 100mL hexane. The product was then dried under high vacuum.

15 The crude product obtained in this manner was azeotroped twice with pyridine and then dissolved in 300mL pyridine. To this was added 8.13g of DMT-Cl and the reaction mixture stirred at room temperature, under argon, for 1.5 hours. The reaction was quenched by the addition of 5mL methanol. The reaction mixture was taken up in 1L methylene chloride, the organic extract washed with 20 5% NaHCO₃ solution, and then dried over anhydrous sodium sulfate. Evaporation of the solvents *in vacuo* afforded 26g of crude product, which was purified by column chromatography over silica gel, eluting with methylene chloride/methanol (100:4 v/v) to yield 5.7g.

Step V: Synthesis of Biotin-CX-CED Phosphoramidite

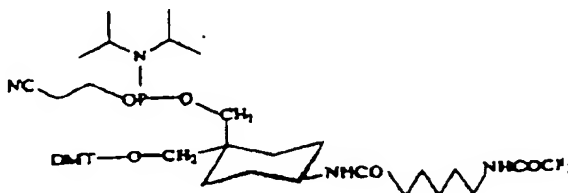
25 The intermediate obtained in step IV above was converted to the corresponding phosphoramidite using standard methods. Thus, 4.0g of DMT-CX-Biotin was dissolved in 40mL methylene chloride and the resulting solution treated with 2.3mL of 2-cyanoethyl-N,N',N'-tetraisopropylphosphorodiamidite and 600mg of DIPA-tetrazole salt. After 15 hours at room temperature, the reaction 30 was quenched by addition of 0.5mL methanol. The reaction mixture was poured into 400mL methylene chloride and the organic layer washed with 5% sodium

-17-

bicarbonate solution (2 X 100mL), and then dried over anhydrous sodium sulfate. Removal of solvents by rotary evaporation gave 5.8g of crude product, which was purified by column chromatography over silica gel, eluted with CH₂Cl₂/methanol/TEA (100:2:1, v/v/v) to yield 3.6g of pure Compound 1.

-18-

- (b) Synthesis of Reagent compound 2 wherein R¹ is dimethoxytriphenyl (DMT), X is O, R² is cyclohexane - NH - CO(CH₂)₅NHCOCF₃, and R³ is phosphoramidite.



[COMPOUND 2]

The synthetic protocol for Compound 2 is outlined below and depicted in Figure 3:

Step I: Synthesis of BzO-CX-Linker

To 15.0g of the NH₂-CX intermediate (prepared in step 2 of Example 1a) dissolved in 150mL methylene chloride, was added dropwise a solution of 21.2g 6-trifluoroacetamido-caproic acid N-hydroxysuccinimide ester (Linker-NHSu) in 150mL methylene chloride. The resulting mixture was stirred at room temperature for 15 min and then treated with 12.1mL triethylamine. After 90 min stirring at room temperature, TLC analysis (CH₂Cl₂/methanol, 9:1) indicated that the reaction had gone to completion.

Methylene chloride was removed by rotary evaporation, the resulting residue treated with 150mL methanol, followed by 30mL of 10% aqueous Na₂CO₃ solution, and the mixture stirred for 1 hour at room temperature. After this time, the reaction mixture was poured into 1.0L CH₂Cl₂ and the organic extract washed with 5% sodium bicarbonate solution. After drying over anhydrous sodium sulfate, the solvents were evaporated *in vacuo* to afford 22g of crude product. Flash

-19-

chromatographic purification using CH_2Cl_2 /methanol (100:3, v/v) as the eluent, afforded 10.6g of pure product.

Step II: Synthesis of DMT-CX-Linker

To a stirred solution of 10.3g BzO-CX-Linker in 100mL DMF, was added
5 15mL of 25% sodium methoxide in methanol, and the resulting mixture stirred at 0-
5°C for 1 hour. The pH of the solution was then adjusted to 7.0 by the addition of
45g Dowex 50X8-100 resin to the reaction mixture followed by stirring for 15 min.
The resin was filtered off and the filtrate evaporated to remove DMF. The
resulting residue was dissolved in 5mL methylene chloride and the product
10 precipitated by the addition of 50mL hexane. The product was then dried under
high vacuum.

The crude product obtained in this manner was azeotroped twice with
pyridine and then dissolved in 100mL pyridine. To this was added 5.9g of
DMT-Cl and the reaction mixture stirred at room temperature, under argon, for 1.5
15 hours. The reaction was quenched by the addition of 3mL methanol and stirred for
30 min. The reaction mixture was taken up in 500mL methylene chloride, the
organic extract washed with 5% NaHCO_3 solution (300mL X 2), and then dried
over anhydrous sodium sulfate. Evaporation of the solvents *in vacuo* afforded
11.9g of crude product, which was purified by column chromatography over silica
20 gel, eluting with methylene chloride/methanol (100:2 v/v) to yield 5.7g.

Step III: Synthesis of N-Linker-CX-CED Phosphoramidite

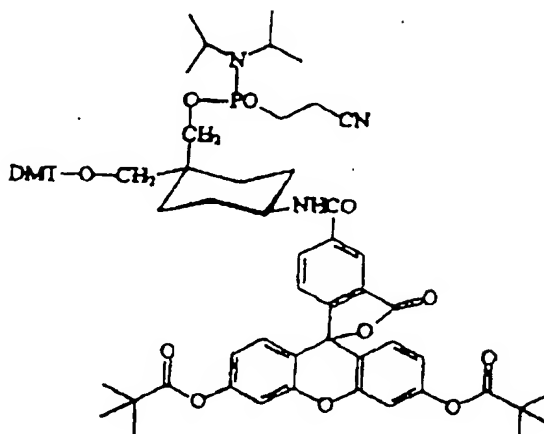
The intermediate obtained in step II above was converted to the
corresponding phosphoramidite using standard methods. Thus, 3.0g of DMT-CX-
Linker was dissolved in 50mL methylene chloride and the resulting solution treated
25 with 2.2mL of 2-cyanoethyl-N,N,N',N'-tetraisopropylphosphorodiamidite and
560mg of DIPA-tetrazole salt. After 15 hours at room temperature, the reaction
was quenched by addition of 1.0mL methanol. The reaction mixture was poured
into 300mL methylene chloride, the organic layer washed with 5% sodium
bicarbonate solution (2 X 80mL), and then dried over anhydrous sodium sulfate.
30 Removal of solvents by rotary evaporation gave 4.3g of crude product, which was

-20-

purified by column chromatography over silica gel, eluted with CH_2Cl_2 /methanol/TEA (100:1:1, v/v/v) - yield 3.1g of pure Compound 2.

-21-

- (c) Synthesis of Reagent compound 3 wherein R¹ is dimethoxytriphenyl (DMT), X is O, R² is cyclohexane - NH - CO - fluorescein, and R³ is phosphoramidite.



[COMPOUND 3]

The synthetic protocol for Compound 3 is outlined below and depicted in Figure 4:

Step I: Synthesis of 4-amino-1,1-bis(hydroxymethyl) cyclohexane

To 30.6g of NH₂-CX dissolved in 400mL of methanol was added 39.3mL of a solution of sodium methoxide (25% w/v) in methanol. The reaction mixture was stirred at ambient temperature for 1 hour under anhydrous conditions. The reaction was monitored by TLC using a mixture of methylene chloride:methanol (9:1) as solvent. Solvents were removed by rotary evaporation and the residue treated with 60mL water, cooled in an ice bath, and then neutralized by the slow addition of hydrochloric acid. The reaction mixture was extracted with methylene chloride (4 X 100mL), and the aqueous portion concentrated *in vacuo* to give 21.5g of product containing sodium chloride. The residue was treated with 200mL methanol, filtered, and solvents removed *in vacuo* to give 13.2g of the desired product.

Step II: 5-(& 6-) Carboxy Fluorescein Dipivaloate

To a solution of 25g 5-(& 6-) carboxy fluorescein in 200mL pyridine, was added 25.8g of diisopropylethyl amine and the resulting mixture cooled to -10°C. To the cooled solution was added dropwise 32.8mL of pivaloyl chloride, and the

-22-

mixture stirred for 2 hours under argon. The reaction mixture was allowed to warm up to room temperature over 2 hours. The reaction mixture was evaporated to dryness, and the residue extracted with 1.0L methylene chloride. The organic extract was washed with water (2 X 500mL), dried over anhydrous sodium sulfate, filtered, and concentrated *in vacuo* to afford 43.4g of crude product. Flash chromatographic purification of this crude product (silica gel, CH₂Cl₂/MeOH, gradient elution 2-8% MeOH) afforded 22.0g of pure product.

Step III: 5-(& 6-)Carboxyfluorescein Dipivaloyl Succinimidyl Ester

To a solution of 28.3g 5-(and 6-) carboxyfluorescein dipivaloate in 250mL methylene chloride, under argon, was added 7.1g N-hydroxysuccinimide followed by 13g of DCC. The reaction mixture was stirred at ambient temperature overnight under anhydrous conditions. The reaction mixture was filtered and the filtrate evaporated to dryness to give 39g of crude product. This product was purified by column chromatography over silica gel, using ethyl acetate/hexane (1:1, v/v) as the eluent to yield 27.4g.

Step IV: Fluorescein-CX-amide

13.2g of 4-amino-1,1-bis(hydroxymethyl)cyclohexane was azeotroped with 50mL of anhydrous DMF using rotary evaporation at 55°C. To this was added 75mL anhydrous DMF followed by 19.5g of 5-(& 6-)carboxyfluorescein dipivaloyl succinimidyl ester under argon. 3.3g of triethylamine was added and the reaction mixture stirred at room temperature overnight under anhydrous conditions. The solution was evaporated to dryness using rotary evaporation at 55±5°C. The residue was extracted into 500mL methylene chloride and the organic extract washed with water (2 X 100mL). After drying over anhydrous sodium sulfate, solvents were removed *in vacuo* to afford 32g of crude product. Flash chromatographic purification of this crude product (silica gel, CH₂Cl₂/MeOH, gradient elution 3-6% MeOH) afforded 7.4g of pure product.

Step V: Synthesis of DMT-CX-Fluorescein

To 7.4g of product obtained from step IV above, was added 50mL of anhydrous pyridine and the mixture azeotroped. The residue was dissolved in 50mL anhydrous pyridine. To this was added 4.6g of DMT-Cl and the reaction

-23-

mixture stirred at room temperature overnight, under argon. The reaction was quenched by the addition of 10mL methanol and stirred for 30 min.

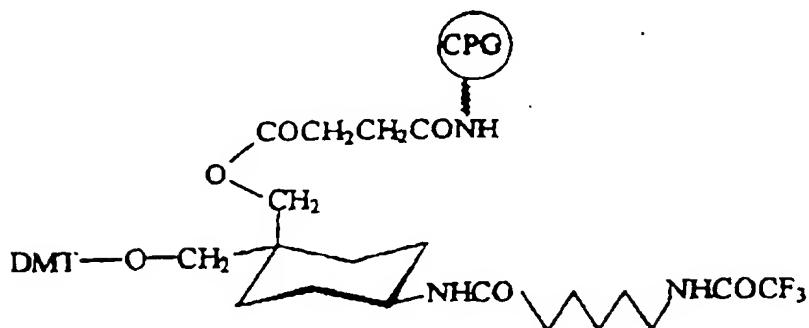
The reaction mixture was taken up in 250mL methylene chloride, the organic extract washed with 75mL of a 5% NaHCO_3 solution, and then dried over anhydrous sodium sulfate. Evaporation of the solvents *in vacuo* afforded 13.5g of crude product, which was purified by column chromatography over silica gel, eluting first with 1.0 L hexane:ethyl acetate (6.5:3.5, v/v) & then with 2.0 L hexane:ethyl acetate (1:1, v/v) to yield 5.8g.

Step VI: Synthesis of Fluorescein-CX-CED Phosphoramidite

The intermediate obtained in step V above was converted to the corresponding phosphoramidite using standard methods. Thus, 5.8g of DMT-CX-Fluorescein was dissolved in 100mL anhydrous methylene chloride and the resulting solution treated with 3.0g of diisopropylethylamine, followed by 1.9g of 2-cyanoethyl N,N-diisopropylchlorophosphoroamidite. After 2 hours at room temperature, the reaction was quenched by addition of 1.0mL methanol. The reaction mixture was poured into 200mL methylene chloride, the organic layer washed with 5% sodium bicarbonate solution (2 X 75mL), and then dried over anhydrous sodium sulfate. Removal of solvents by rotary evaporation gave 8.0g of crude product, which was purified by column chromatography over silica gel, eluted with hexane/ethyl acetate/ TEA (gradient elution - 30.0 to 35.0 % ethyl acetate in hexane containing 0.5 % TEA) to yield 4.2g of pure Compound 3.

-24-

- (d) Synthesis of Reagent compound 4 wherein R¹ is dimethoxytriphenyl (DMT), X is O, R² is cyclohexane - NH - CO(CH₂)₅NHCOCF₃, and R³ is -OCOCH₂CH₂CONH - CPG.



[COMPOUND 4]

The synthetic protocol for Compound 4 is outlined below:

Step 1: Preparation of N-Linker succinate:

To a stirred solution of 1.95g of DMT-N-Linker-CX in 20mL anhydrous methylene chloride, was added 100mg of 4-dimethylaminopyridine followed by 1.2g of succinic anhydride. The resulting solution was stirred at room temperature for 15 hours. The reaction mixture was quenched by the addition of 10mL of a 5% solution of sodium bicarbonate in water and the mixture stirred for 30 min. The crude reaction mixture was then evaporated to dryness *in vacuo* and the resulting residue extracted twice with 50mL of methylene chloride.

The organic extract was washed with 5% aqueous citric acid solution (2 X 20mL) and then dried over anhydrous sodium sulfate. Evaporation of the solvents *in vacuo* afforded 1.9g of the crude product. This product was purified by flash chromatography over silica gel using CH₂Cl₂/CH₃OH (100:5, v/v) as the eluant.

Step 2: Preparation of N-Linker-CPG:

To a suspension of 4.0g of LCAA-CPG in 14mL of methylene chloride in a 50mL round bottomed flask, was added 105mg of N-Linker succinate and 0.7mL of triethylamine. This was followed by the addition of 20mg of anhydrous

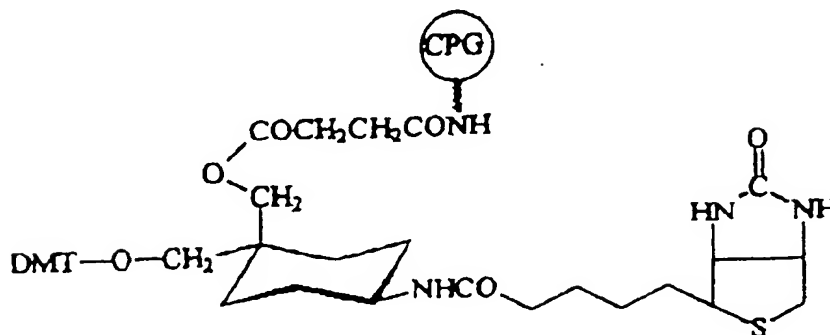
-25-

hydroxybenzotriazole and 70mg of benzotrizolyl-N-oxy-tris(dimethylamino) phosphonium hexafluorophosphate (BOP reagent).

5 The resulting mixture was gently shaken for 2 hours, then filtered, washed with methylene chloride (10mL X 2), and air dried. The solid was transferred to a 100mL round bottom flask, treated with 36mL pyridine, 4mL of acetic anhydride and 0.4mL of N-methylimidazole, and the resulting suspension shaken overnight. The mixture was then suction filtered, and the solid washed with methanol (10mL X 3). The solid was washed further with methylene chloride (10mL X 3), followed by anhydrous ether (10mL X 3). The solid was air dried and then finally dried
10 overnight under high vacuum.

-26-

- (e) Synthesis of Reagent compound 5 wherein R^1 is dimethoxytriphenyl (DMT), X is O, R^2 is cyclohexane - NH - CO - biotin, and R^3 is -OCOCH₂CH₂CONH - CPG.



[COMPOUND 5]

The synthetic protocol for Compound 5 is illustrated in Figure 5, and outlined below:

Step 1: Preparation of Biotin succinate:

To a stirred solution of 2.0g of DMT-CX-Biotin in 20mL anhydrous methylene chloride, was added 100mg of 4-dimethylaminopyridine followed by 1.2g of succinic anhydride. The resulting solution was stirred at room temperature for 15 hours at which point TLC analysis of the reaction mixture (9:1 CH₂Cl₂/CH₃OH) indicated that the reaction was complete.

The reaction mixture was quenched by the addition of 10mL of a 5% solution of sodium bicarbonate in water and the mixture stirred for 30 min. The crude reaction mixture was then evaporated *in vacuo* and the resulting residue extracted twice with 50mL of methylene chloride. The organic extract was washed with 5% aqueous citric acid solution (20mL X 2) and then dried over anhydrous sodium sulfate. Evaporation of the solvents in *vacuo* afforded 2.4g of the crude product. This product was purified by flash chromatography over silica gel using CH₂Cl₂/CH₃OH (100:4, v/v) as the eluant.

-27-

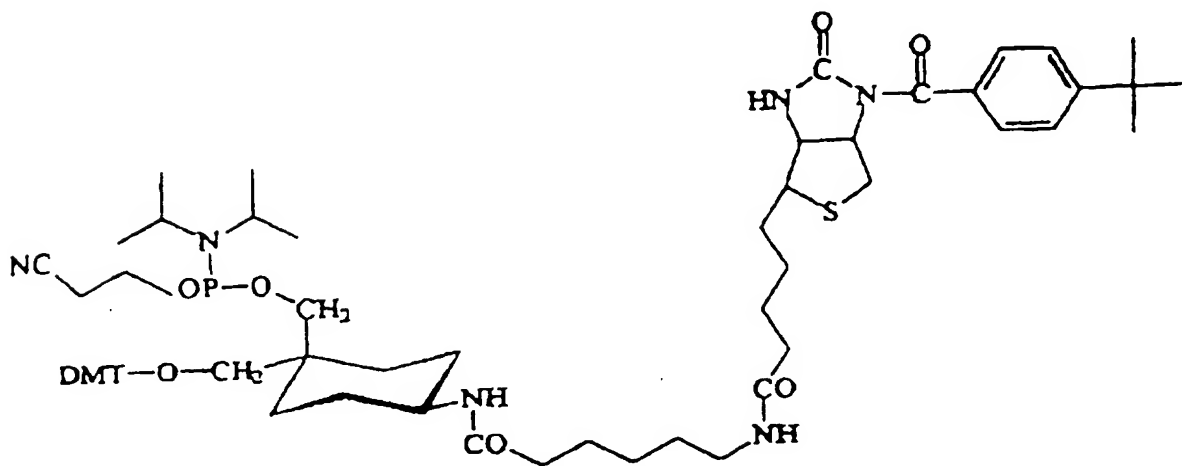
Step 2: Preparation of Biotin-CPG:

To a suspension of 4.0g of LCAA-CPG in 14mL of methylene chloride in a 50mL round bottomed flask, was added 110mg of Biotin succinate and 0.7mL of triethylamine. This was followed by the addition of 20mg of anhydrous hydroxybenzotriazole and 70mg of BOP reagent. The resulting mixture was gently shaken for 2 hours, then filtered, washed with methylene chloride (20mL X 2), and air dried. The solid was transferred to a 100mL round bottom flask, treated with 36mL pyridine, 4mL of acetic anhydride and 0.4mL of N-methylimidazole, and the resulting suspension shaken overnight.

The mixture was then suction filtered, and the solid washed with methanol (10mL X 3). The solid was washed further with methylene chloride (10mL X 3), followed by anhydrous ether (10mL X 3). The solid was air dried and then finally dried overnight under high vacuum. The loading of the biotin derivatized CPG was determined to be 31.3 μ mole/gram using standard methods.

-28-

- (f) Synthesis of Reagent compound 6 wherein R^1 is dimethoxytriphenyl (DMT), X is O, R^2 is cyclohexane - NH - CO(CH₂)₅NHCO - biotin, and R^3 is phosphoramidite.



[COMPOUND 6]

The synthetic protocol for Compound 6 is outlined below and depicted in Figure 5:

Step I: Synthesis of BzO-CX-Biotin-LC

To 32.0g of the NH₂-CX intermediate (prepared as discussed in Example 1a) dissolved in 500mL anhydrous methylene chloride, was added dropwise a solution of 39.5g LC Biotin-NHSu (prepared by the reaction of 6-aminocaproic acid with the NHSu-ester of biotin) in 500mL anhydrous DMF. The resulting mixture was stirred at room temperature for 15 min and then treated with 12.1mL triethylamine. After 2 hours stirring at room temperature, TLC analysis (CH₂Cl₂/methanol, 9:1) indicated that the reaction had gone to completion. Methylene chloride was removed by rotary evaporation, the resulting solution treated with methanol (20mL) followed by 10% aqueous Na₂CO₃ (20mL), and the mixture stirred for 1 h at room temperature. After this time, the reaction mixture was poured into 2.0 L ethyl acetate and the organic extract washed with brine (500mL X 2). After drying over anhydrous sodium sulfate, the solvents were evaporated *in vacuo* to afford 64g of crude product. Flash chromatographic purification of this crude product (silica gel, CH₂Cl₂/MeOH, gradient elution 5-8% MeOH) afforded 30g of pure product.

Step II: Synthesis of DMT-CX-Biotin-LC

To a stirred solution of 29.8g of BzO-CX-Biotin-LC in 300mL DMF, was added 34.5mL of 25% sodium methoxide in methanol, and the resulting mixture stirred at 0-5°C for 1 hour. The pH of the solution was then adjusted to 7.0 by the addition of 100g Dowex 50X8-100 resin to the reaction mixture followed by stirring for 15 min. The resin was filtered off and the filtrate evaporated to remove DMF. The resulting residue was dissolved in 60mL methylene chloride and the product precipitated by the addition of 200mL hexane. The product was then dried under high vacuum.

The crude product obtained in this manner was azeotroped twice with pyridine and then dissolved in 500mL pyridine. To this was added 14.0g of DMT-Cl and the reaction mixture stirred at room temperature, under argon, for 1.5 hours. The reaction was quenched by the addition of 5mL methanol and stirred for 30 min. The reaction mixture was taken up in 1.5L methylene chloride, the organic extract washed with 5% NaHCO₃ solution (500mL X 2), and then dried over anhydrous sodium sulfate. Evaporation of the solvents *in vacuo* afforded 26.0g of crude product, which was purified by column chromatography over silica gel, eluting with methylene chloride/methanol (100:8 v/v) to yield 12.0g.

Step III: Synthesis of 'BuBz-DMT-CX-Biotin-LC

To a stirred solution of 12.0g of DMT-CX-Biotin in 300mL anhydrous pyridine, was added 13.0mL TMSCl and the mixture stirred at room temperature for 2 hours, under argon. This was followed by the addition of 4.4mL of 4-tert-butyl benzoyl chloride to the reaction mixture and the reaction was allowed to proceed at room temperature for 3 hours. The reaction was worked up by the addition of 80mL water followed by stirring for 1 hour at room temperature. The reaction mixture was evaporated to remove most of the pyridine and the resulting residue dissolved in 1.0L methylene chloride. The organic extract was washed with 5% NaHCO₃ solution (300mL X 2), and then dried over anhydrous sodium sulfate. Removal of solvents by rotary evaporation gave 18.3g of crude product.

-30-

Flash chromatographic purification of this crude product (silica gel, EtOAc/CH₂Cl₂/MeOH, gradient elution with solutions containing 5-10 parts MeOH in EtOAc/ CH₂Cl₂, 50:50 parts, v/v/v) afforded 8.3g of pure product.

Step IV: Synthesis of LC-Biotin-CX-CED Phosphoramidite

- 5 The intermediate obtained in step III above was converted to the corresponding phosphoramidite using standard methods. Thus, 8.2g of 'BuBz-DMT-CX-Biotin-LC was dissolved in 100mL methylene chloride and the resulting solution treated with 4.0mL of 2-cyanoethyl-N,N,N',N'-
- 10 tetraisopropylphosphorodiamidite and 600mg of DIPA-tetrazole salt. After 15 hours at room temperature, the reaction was quenched by addition of 0.5mL methanol. The reaction mixture was poured into 1.0L methylene chloride, the organic layer washed with 5% sodium bicarbonate solution (2 X 300mL), and then dried over anhydrous sodium sulfate. Removal of solvents by rotary evaporation
- 15 gave 10.5g of crude product, which was purified by column chromatography over silica gel, and eluted with CH₂Cl₂/methanol/TEA (100:1:1, v/v/v) to yield 5.0g of pure Compound 6.

Example 2

Reagents in accordance with the present invention can be incorporated into oligomers comprising nucleotide and non-nucleotide units, by substituting the present non-nucleotide reagents in place of selected nucleotide units in standard nucleotide synthesis protocols, such as automated DNA/RNA synthesis protocols.

(a) **Use of Biotin-CX by direct binding to polystyrene plates:**

Synthetic oligomers, containing one or more biotin residues as part of the sequence, were labelled with a 5'-phosphate group. This 5'-phosphate moiety is then used to covalently bind the oligomer onto polystyrene microtiter plates.

The unbound probes are washed off and the bound probes are detected by the reaction of biotin with streptavidin conjugated to alkaline phosphatase. The alkaline phosphatase catalyses the hydrolysis of a chromogenic substrate.

Thus, 30mers were synthesized which included a 5' phosphate moiety and biotin residue labels at positions 13, 19 and 25 (5' → 3'). Two different oligomers were synthesized: one with Biotin-CX phosphoramidite prepared in accordance with Example 1 and the second oligomer with Biotin-dC (a commercially available nucleosidic biotin phosphoramidite).

Each of the oligomers was diluted to a concentration of 10fmol/ μ L in distilled water. The oligomers were denatured by heating at 95°C for 10 min, followed by cooling over ice for 10 min. The appropriate amount of denatured oligomers were added to the wells of cold Covalink NH modules, followed by the addition of EDC buffer containing Melm and then overnight incubation at 50°C. Unbound probes were washed off and the bound oligomers were detected by binding of streptavidin conjugated to alkaline phosphatase. pNPP was used as the substrate for the enzyme and the development of color was monitored at 405nm. The above experiment was repeated using both oligomers at a concentration of 5fmol/ μ L. The signal intensities obtained using the two different biotin structures were compared. At 5fmol/ μ L, biotin-CX gave 95% of the reading (OD 405nm) of biotin-dC. At 10fmol/ μ L, biotin-CX gave 106% of the reading of biotin-dC.

-32-

(b) Use of Biotin-CX in a hybridization assay:

The oligomer Alu-011 is a 56mer with one internal biotin residue at position 25 (5' → 3'), designed to be complementary to the template Alu-011A. Two different oligomers were synthesized: One with Biotin-CX and the other with Biotin-dC. Oligomers were synthesized in the Trityl-ON mode and then cartridge purified using PolyPak (Glen Research Inc.) reverse phase cartridges. These two oligomers were used in hybridization assays to detect the template Alu-011A.

The template Alu-011A was bound to CovaLink polystyrene microtiter plates using the 5'-phosphate group as described above. The probes were diluted to 25fmol/ μ L in hybridization buffer and 100 μ L of the diluted probes was added to the wells and incubated at 42°C for from 5 hours to overnight. Excess probes were washed off with buffer and the bound biotin-labelled probes were detected as described above.

The signal intensities obtained using the two different biotin structures were compared. Under identical conditions, Biotin-CX labelled oligomers generated approximately a 5-fold stronger signal than oligomers labelled with Biotin-dC.

All publications and patent applications cited in this specification are hereby incorporated by reference as if they had been specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity and understanding, it will be apparent to those of ordinary skill in the art in light of the disclosure that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

-33-

Claims:

1. A non-nucleotide reagent which is capable of forming a oligomer with nucleotide units, said reagent comprising a compound of the formula:

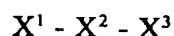


wherein

R^1 is selected from the group consisting of hydrogen, acid-sensitive, base-stable blocking groups and acyl capping groups;

X is selected from the group consisting of O, S, NH and N=N;

R^2 is a substituent group of the formula



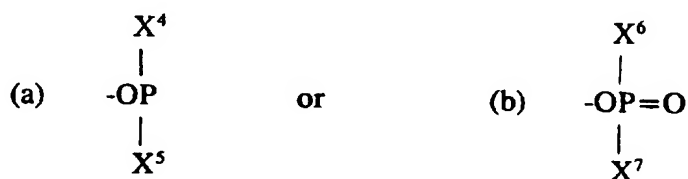
wherein

X^1 is a substituted or unsubstituted C_5 to C_7 cyclic moiety incorporating the carbon atom of the formula;

X^2 is selected from the group consisting of O, S, CH_2 , NH and N=N; and

X^3 is hydrogen, or a linking functional group which is capable of linking with a functional moiety; and

R^3 is a phosphorus linking group of the formula



wherein

X^4 is halogen or substituted amino,

X^5 is alkyl, alkoxy or phenoxy, or a cyano derivative thereof,

X^6 is halogen, amino or O, and

X^7 is alkyl, alkoxy or aryloxy, or may be H only if X^6 is O, or

R^3 is a bond, either directly or through an intermediate group, to a solid support.

-34-

2. A reagent as recited in claim 1, wherein R¹ is selected from the group consisting of triphenylmethyl compounds and alkoxy derivatives thereof.

3. A reagent as recited in claim 2, wherein R¹ is dimethoxytriphenyl.

4. A reagent as recited in claim 1, wherein X is O.

5. A reagent as recited in claim 1, wherein X¹ is cyclohexane.

6. A reagent as recited in claim 1, wherein X² is NH.

7. A reagent as recited in claim 1, wherein X³ is a linking group of the formula



wherein n is an integer from 0 to 20.

8. A reagent as recited in claim 1, wherein X³ further comprises a functional moiety.

9. A reagent as recited in claim 8, wherein the functional moiety is selected from the group consisting of labels, metal chelators and specific binding agents.

10. A reagent as recited in claim 9, wherein the functional moiety is a fluorescent label.

11. A reagent as recited in claim 10, wherein the fluorescent label is fluorescein.

12. A reagent as recited in claim 9, wherein the functional moiety is a specific binding agent.

-35-

13. A reagent as recited in claim 12, wherein the specific binding agent is biotin.

5 14. A reagent as recited in claim 1, wherein R^3 is selected from the group consisting of phosphodiester, phosphotriester, phosphite, phosphoramidite, H-phosphonate, alkyl-phosphonate, and phosphorothioate.

10 15. A reagent as recited in claim 1, wherein R^3 comprises a bond, either directly or through an intermediate group, to a solid support.

16. A reagent as recited in claim 15, wherein the intermediate group comprises $-O-CO(CH_2)_2-CO-$.

-36-

17. An oligomer having both nucleotide and non-nucleotide units, at least one of said non-nucleotide units comprising a compound of the formula:

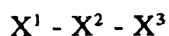


wherein

5 R^1 is selected from the group consisting of hydrogen, acid-sensitive, base-stable blocking groups and acyl capping groups, or R^1 is a bond to an adjacent monomeric unit;

X is selected from the group consisting of O, S, NH and N=N;

R^2 is a substituent group of the formula



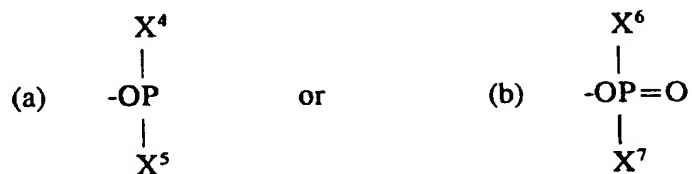
wherein

X^1 is a substituted or unsubstituted C_5 to C_7 cyclic moiety incorporating the carbon atom of the formula;

15 X^2 is selected from the group consisting of O, S, CH_2 , NH and N=N; and

X^3 is hydrogen, or a linking functional group which is capable of linking with a functional moiety; and

R^3 is a phosphorus linking group of the formula



25 wherein

X^4 is halogen or substituted amino,

X^5 is alkyl, alkoxy or phenoxy, or a cyano derivative thereof,

X^6 is halogen, amino or O, and

X^7 is alkyl, alkoxy or aryloxy, or may be H only if X^6 is O,

30 or R^3 is a bond to an adjacent monomeric unit or directly or through an intermediate group a solid support,

with the proviso that at least one of R^1 and R^3 is a bond.

-37-

18. A method for preparing an oligomer having both nucleotide and non-nucleotide units, comprising

coupling at least one non-nucleotide unit comprising a compound of the formula:

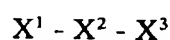


wherein

R^1 is selected from the group consisting of hydrogen, acid-sensitive, base-stable blocking groups and acyl capping groups, or R^1 is a bond to an adjacent monomeric unit;

10 X is selected from the group consisting of O, S, NH and N=N;

R^2 is a substituent group of the formula



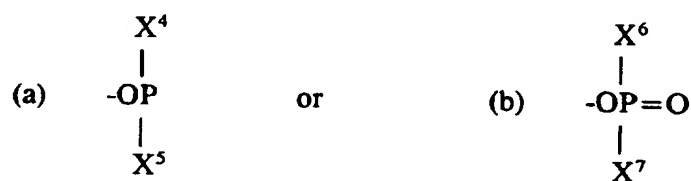
wherein

15 X^1 is a substituted or unsubstituted C_5 to C_7 cyclic moiety incorporating the carbon atom of the formula;

X^2 is selected from the group consisting of O, S, CH_2 , NH and N=N; and

X^3 is hydrogen, or a linking functional group which is capable of linking with a functional moiety; and

20 R^3 is a phosphorus linking group of the formula



wherein

X^4 is halogen or substituted amino,

X^5 is alkyl, alkoxy or phenoxy, or a cyano derivative thereof,

30 X^6 is halogen, amino or O, and

X^7 is alkyl, alkoxy or aryloxy, or may be H only if X^6 is O, or

-38-

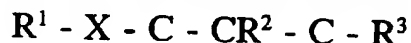
R^3 is a bond, either directly or through an intermediate group, to an adjacent monomeric unit or a solid support,
to at least one nucleotide monomeric unit by a bond at either R^1 or R^3 .

-39-

19. A kit for preparing an oligomer having both nucleotide and non-nucleotide units, comprising

a receptacle adapted to hold one or more individual reagent containers; and

5 a first container containing a reagent in accordance with the formula:

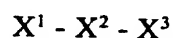


wherein

R^1 is selected from the group consisting of hydrogen, acid-sensitive, base-stable blocking groups and acyl capping groups;

10 X is selected from the group consisting of O, S, NH and N=N;

R^2 is a substituent group of the formula



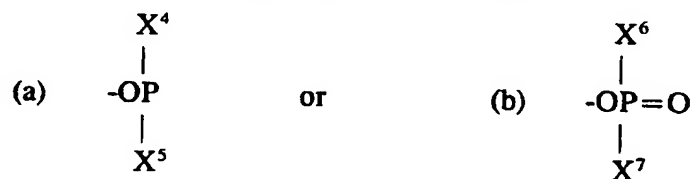
wherein

15 X^1 is a substituted or unsubstituted C_5 to C_7 cyclic moiety incorporating the carbon atom of the formula;

X^2 is selected from the group consisting of O, S, CH_2 , NH and N=N; and

X^3 is hydrogen, or a linking functional group which is capable of linking with a functional moiety; and

20 R^3 is a phosphorus linking group of the formula



wherein

X^4 is halogen or substituted amino,

X^5 is alkyl, alkoxy or phenoxy, or a cyano derivative thereof,

30 X^6 is halogen, amino or O, and

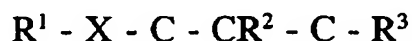
X^7 is alkyl, alkoxy or aryloxy, or may be H only if X^6 is O, or

R^3 is a bond, either directly or through an intermediate group, to a solid support.

-40-

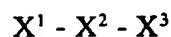
20. A kit as recited in claim 19, further comprising a second container containing (1) a reagent used in the synthesis of oligomers, or (2) a reagent used in the detection of a functional moiety associated with said reagent.

5 21. A chemical intermediate for synthesizing a non-nucleotide reagent which is capable of forming a oligomer with nucleotide units, said intermediate comprising a compound of the formula:



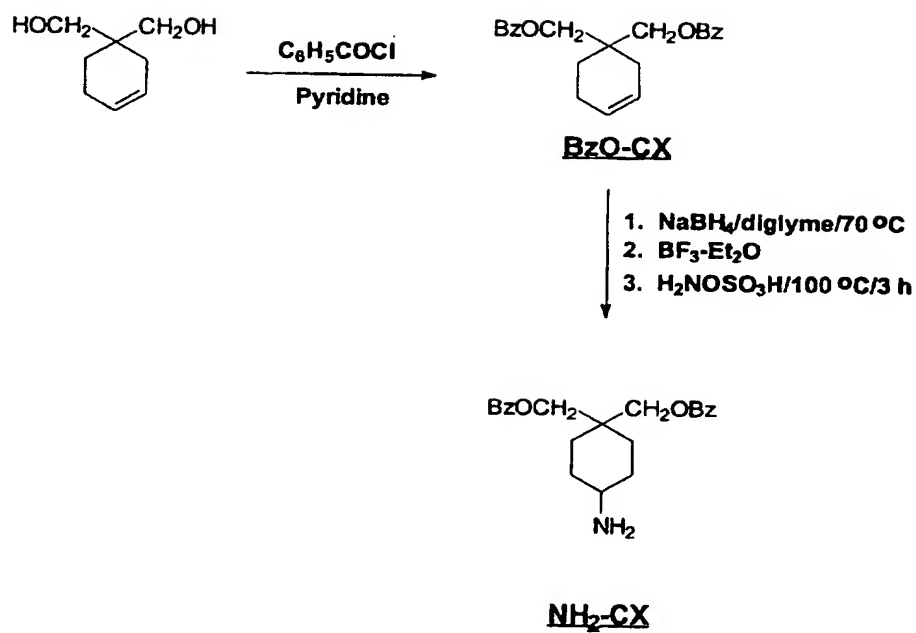
wherein

10 R^1 is hydrogen;
 X is oxygen;
 R^2 is a substituent group of the formula



wherein

15 X^1 is cyclohexane,
 X^2 is NH, and
 X^3 is H; and
 R^3 is OH.

Scheme 1a:**Figure 1**

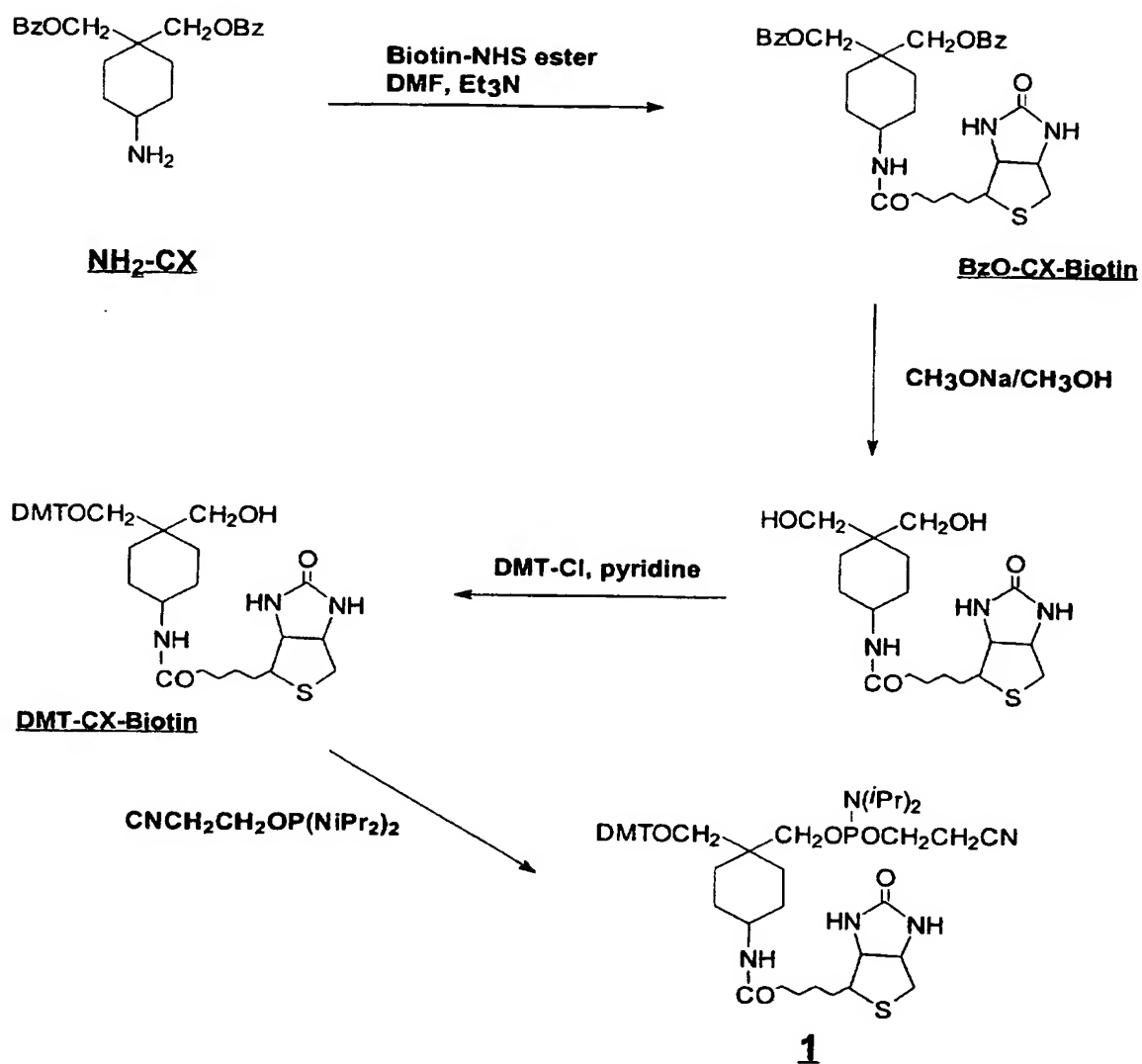
Scheme 1b: Synthesis of compound 1

Figure 2

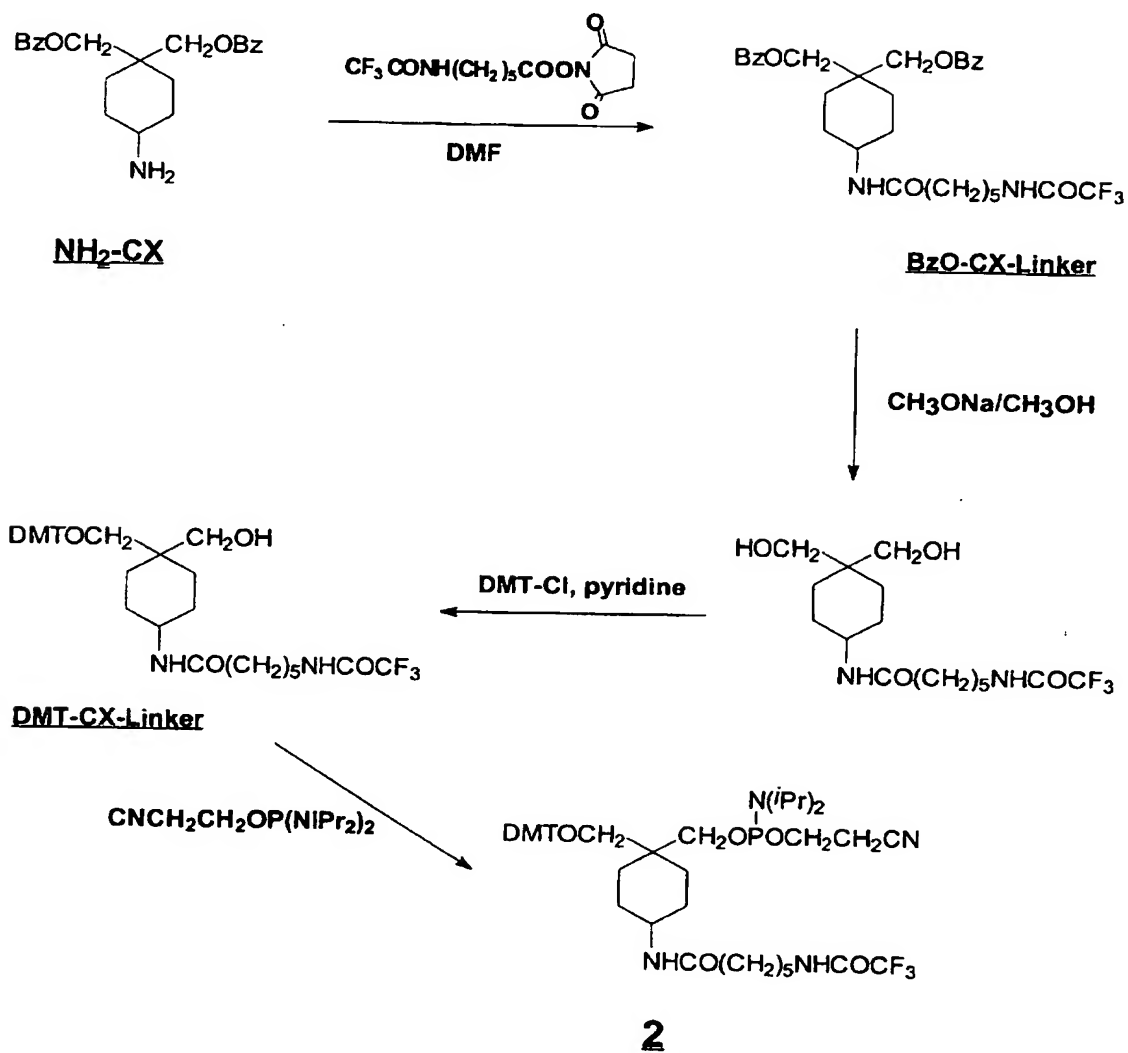
Scheme 2: Synthesis of compound 2

Figure 3

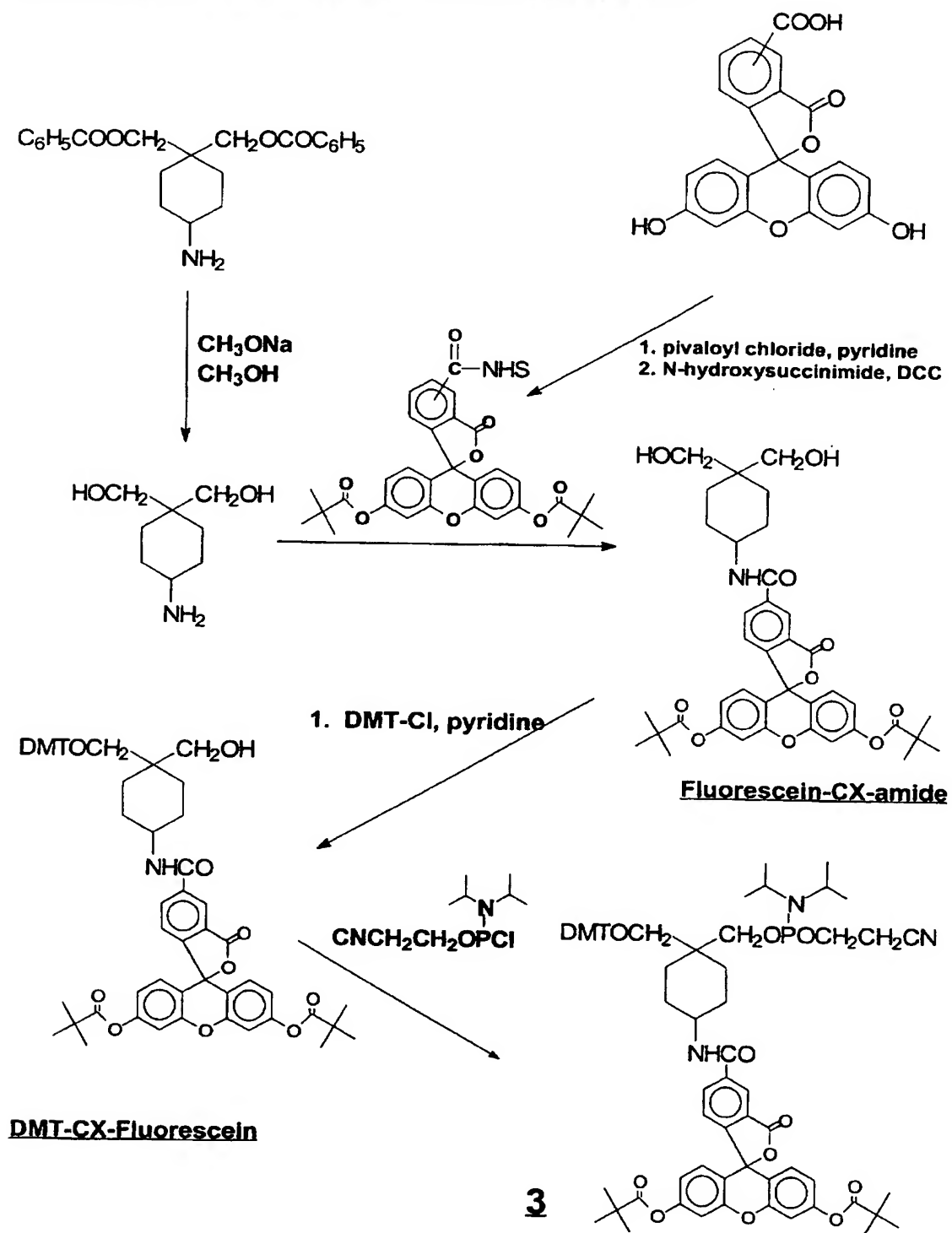
Scheme 3: Synthesis of compound 3

Figure 4

SUBSTITUTE SHEET (RULE 26)

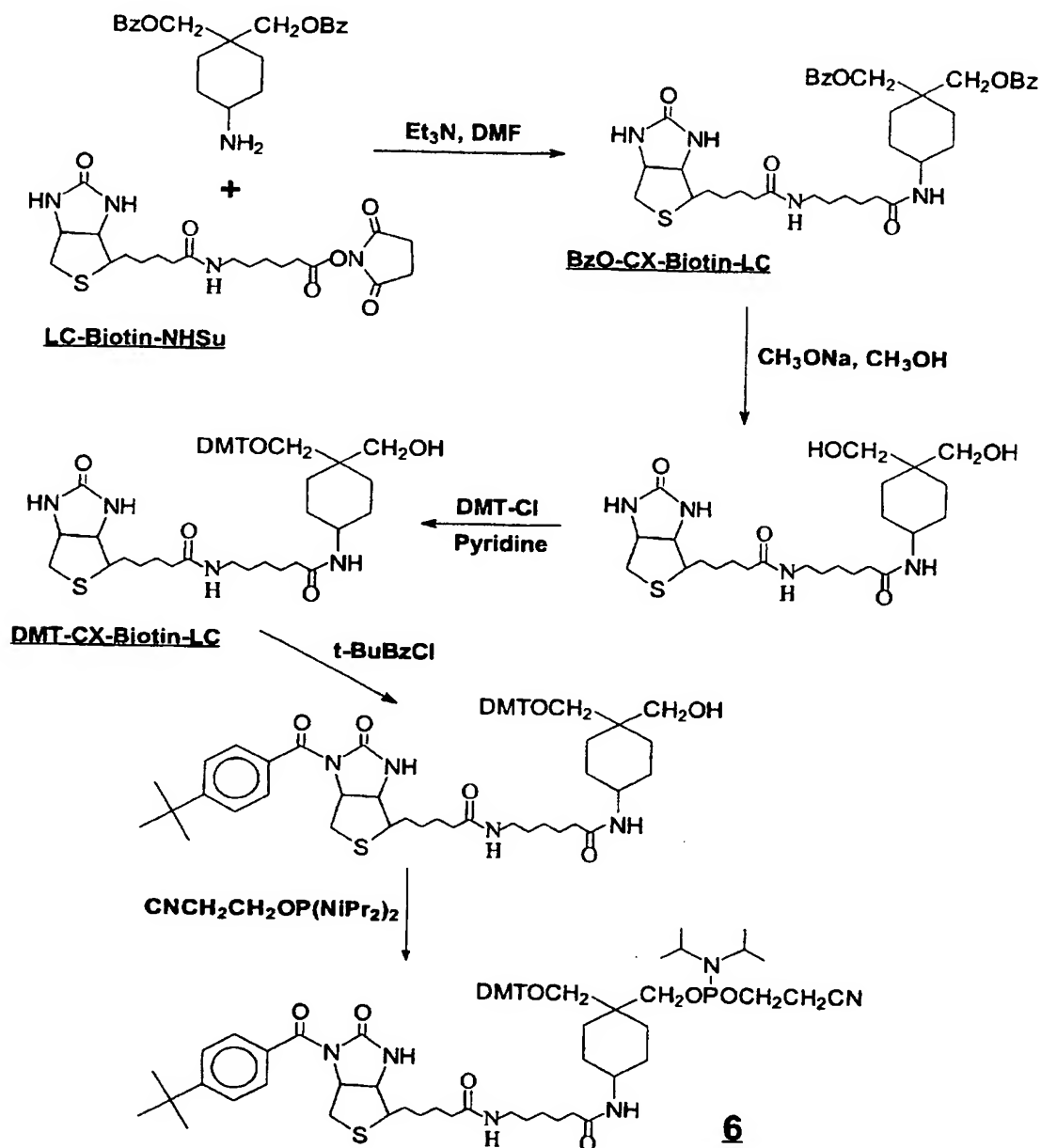
Scheme 4: Synthesis of compound 6

Figure 5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/09094

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12Q 1/68; C07H 19/00, 21/00, 21/02, 21/04

US CL : 435/6; 536/22.1, 23.1, 25.3

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6; 536/22.1, 23.1, 25.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, STN

search terms: phosphoramidite, nucleotide, oligomer, linker, solid support, cyclohexane, label

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SKARIC et al. Synthesis and Properties of the Stereoisomeric Diethyl 2-Aminocyclohexane-1,4-Dicarboxylates. Journal Chemical Society Perkins I. 1974, pages 1406-1410, especially page 1406.	21
X	SKARIC et al. Synthesis and Properties of the Stereoisomeric Diethyl 6-Aminocyclohexane-1,3-Dicarboxylates. Journal Chemical Society Perkins I. 1975, pages 1959-1961, especially page 1959.	21

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

Special categories of cited documents:	
A document defining the general state of the art which is not considered to be of particular relevance	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
E earlier document published on or after the international filing date	*X* document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Y* document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
O document referring to an oral disclosure, use, exhibition or other means	*A* document member of the same patent family
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

05 AUGUST 1997

Date of mailing of the international search report

03 SEP 1997

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

JEZIA RILEY

Telephone No. (703) 308-0196